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Research article

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Coupling of importin beta binding peptide on plasmid DNA: transfection efficiency is increased by modification of lipoplex's physico-chemical properties

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Abstract

Background: Non-viral vectors for gene transfer are less immunogenic than viral vectors but also less efficient. Significant effort has focused on enhancing non-viral gene transfer efficiency by increasing nuclear import of plasmid DNA, particularly by coupling nuclear localization peptidic sequences to plasmid DNA.

Results: We have coupled a 62-aminoacid peptide derived from hSRP1 α importin beta binding domain, called the IBB peptide to plasmid DNA by using the heterobifunctional linker N-(4-azido-2,3,5,6 tetrafluorobenzyl)-6-maleimidyl hexanamide (TFPAM-6). When covalently coupled to plasmid DNA, IBB peptide did not increase the efficiency of cationic lipid mediated transfection. The IBB peptide was still able to interact with its nuclear import receptor, importin β , but non-specifically. However, we observed a 20-fold increase in reporter gene expression with plasmid DNA / IBB peptide complexes under conditions of inefficient transfection. In which case, IBB was associated with plasmid DNA through self assembling ionic interaction.

Conclusions: The improvement of transfection activity was not due to an improved nuclear import of DNA, but rather by the modification of physicochemical properties of IBB peptide / plasmid complexes. IBB peptide increased lipoplex size and these larger complexes were more efficient for gene transfer.

Background

Even though lipofection represents 13 % of gene therapy clinical trials, cationic lipids remain less efficient gene delivery vectors than viral vectors. The first obstacle in *in vivo* non-viral gene transfer is the transport of plasmid DNA from the site of injection to the targeted cell: lipoplexes have to circulate without being sequestered by the

reticulo-endothelial system [1] in order to reach the targeted cell membrane. Lipoplexes then encounter physiological barriers to their intracellular trafficking. After cell membrane attachment, lipoplexes enter the cell cytoplasm either by fusion of the vector with the plasma membrane or by endocytosis [2], and then inefficiently diffuse through the cytoplasm to the nuclear envelope [3].

Plasmid DNA carrying the therapeutic gene most frequently gains access to the nucleus during mitosis, when nuclear envelope rupture occurs [4,5]. In the case of non-dividing cells, transfection is particularly low, presumably because of the diffusion barrier constituted by the nuclear envelope [5]. Consequently, much work has been done to increase the efficiency of gene transport to the cell nucleus. Several authors have worked on strategies mimicking viral genome or karyophilic protein nuclear delivery. Proteins destined to the nucleus possess at least one nuclear localisation sequence (NLS) which allows them to interact with a nuclear import receptor, namely importin β (for a review, see [6]). When complexed to importin β , proteins are delivered to cell nuclei via nuclear pore complexes. Most proteins interact with importin β via an adapter, importin α . Importin α consists of two functional domains, a short basic amino-terminal domain responsible for importin β binding (IBB domain) and a central NLS-binding domain built of armadillo (arm) repeats [7–9]. Nuclear localization sequences, and among them SV40 large T antigen NLS sequence, have been electrostatically [10–13] or covalently [14–17] coupled to plasmid DNA to provide an interaction with importin α . The hypothesis was that plasmid DNA / importin α complexes would then interact with importin β and be transported to the nucleus by a facilitated diffusion pathway via the protein nuclear import system. It has been reported that covalent coupling of NLS peptides to circular plasmid DNA leads to nuclear accumulation if more than one hundred peptides per plasmid are coupled [14]. Nevertheless, such NLS peptide-plasmid DNA chimera are not transcribed and thus not biologically active. We have previously described functional plasmids covalently coupled to a limited amount of NLS sequences, which have been shown to interact with importin α [15]. However, such modified plasmids did not accumulate in cell nuclei [15]. Similarly, when a single NLS peptide is covalently coupled to plasmid DNA, nuclear accumulation is not observed [17], except in the case where plasmid DNA is highly engineered. For instance, Zanta et al. linearized plasmid DNA, capped it and coupled a single NLS peptide on one cap. With this construction, transfection efficiency was enhanced in several cell types [16].

These strategies require the contribution of the importin α adapter, which could reduce the efficiency of plasmid DNA nuclear targeting. Subramanian et al. coupled the M9 domain of hnRNP A1 to plasmid DNA via electrostatic interactions mediated by a covalently coupled scrambled SV40 T antigen NLS [18]. The M9 domain is known to interact directly with transportin [19], a member of the karyopherin β import receptor family. With this construction, reporter gene expression was increased by 63-fold. However, a nuclear export signal (NES) is enclosed in the M9 domain and could provide its nuclear

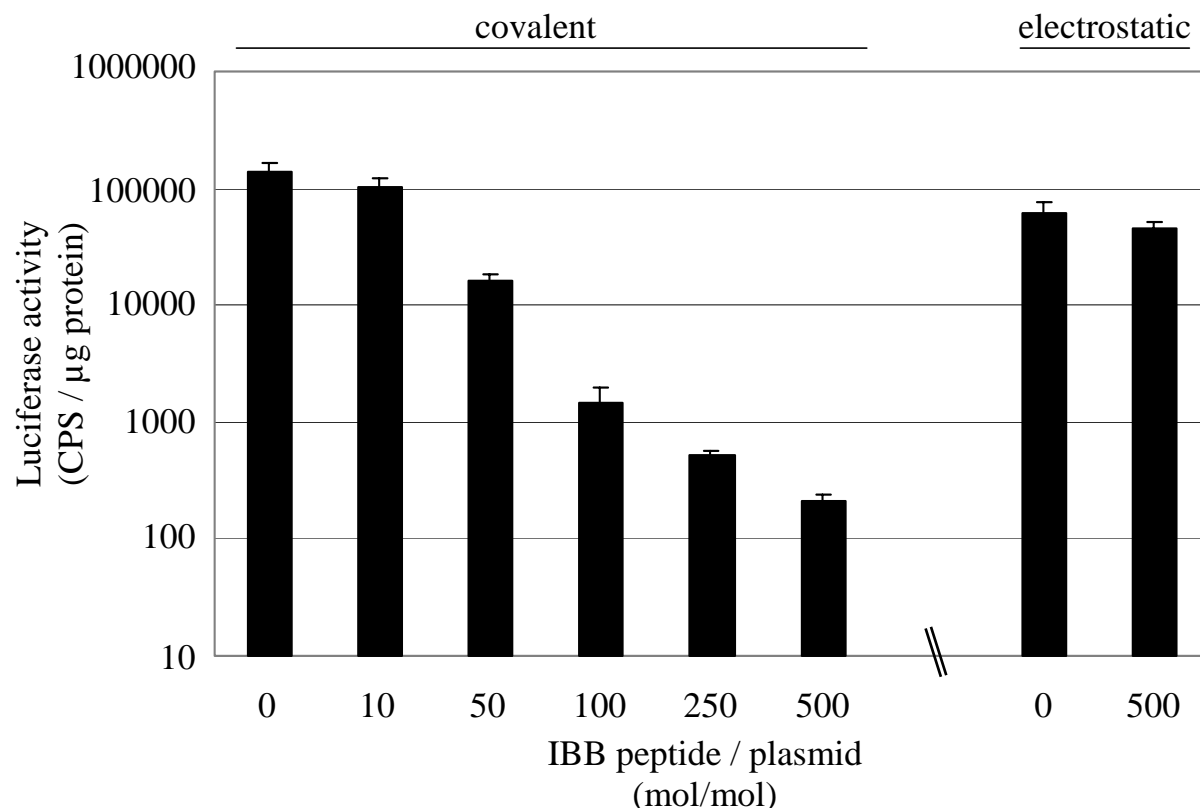
externalization. We hypothesized that the covalent coupling of the importin α IBB domain to plasmid DNA could mediate direct interaction between plasmid and importin β , without the need of an adapter. Human importin α 2 (hSRP1 α) IBB domain has been described by Weis et al. [20]. Its structure has been shown to be modular, with separate domains having separate functions. Particularly, the 1–51 N-terminal sequence of hSRP1 α has been shown to be sufficient for importin β interaction, with residues 21–51 being particularly important, and residues 1–21 further enhancing binding to importin β [21]. No nuclear export signal has been reported in the IBB sequence.

In the present work, our first strategy was to covalently couple the IBB peptide hSRP1 α 11–54 amino acids to plasmid DNA. It was the first time that the IBB domain of importin α was used as an additive in the formulation of lipoplexes. We tested whether covalent coupling of IBB peptide to plasmid DNA increased *in vitro* transfection via enhanced nuclear import. DNA-coupled IBB was still able to interact with importin β , also non-specifically and no improvement of gene transfer was observed. Nevertheless, we observed an increased reporter gene expression using non-covalent plasmid DNA / IBB peptide self assembling complexes. This enhancement was attributed more to changes in the lipoplex's physico-chemical properties than to nuclear targeting of plasmid DNA by the IBB peptide.

Results

Cationic lipid mediated transfection of plasmid DNA-IBB peptide covalent chimera

The IBB peptide was covalently coupled to plasmid DNA by photoactivation at various IBB peptide / plasmid molar ratios. We first tried to evaluate the direct effect of IBB peptide on nuclear import using transfection since our final aim was to enhance gene transfer by transfection. When using RPR120535 cationic lipid to mediate plasmid-IBB peptide chimera transfection, we observed a partial dose-dependent inhibition of reporter gene expression, which reached 88 % at 50 IBB peptide / plasmid (mol / mol), and 99 % at 100 IBB peptide / plasmid (mol / mol) (Figure 1). At 1 to 10 molar ratios, reporter gene expression following transfection was neither inhibited, nor increased (data not shown). The same result was obtained using confluent cells, i.e. non-dividing cells (data not shown). Thus the plasmid DNA was still biologically active but transfection efficiency was not enhanced by covalently coupled IBB peptides. At high IBB peptide / plasmid ratios, the inhibition of transfection was a consequence of the photoactivation as non-photoactivated IBB / plasmid complexes of the same high ratio exhibited the same transfection activity as the control plasmid (Figure 1). In an effort to determine why covalent coupling of IBB

**Figure 1**

Transfection of HeLa cells using a ternary lipoplexe IBB peptide / plasmid / RPRI20535 cationic lipid. Lipoplexes were prepared in bicarbonate buffer (150 mM NaCl, 20 mM NaHCO₃, pH 10) and diluted with serum-containing cell culture medium. The peptide was covalently coupled (covalent) or electrostatically associated (electrostatic) to pXL3031 luciferase-encoding plasmid at a ratio varying from 0 to 500 IBB peptide / plasmid (mol / mol). Luciferase expression was evaluated 24 h after transfection.

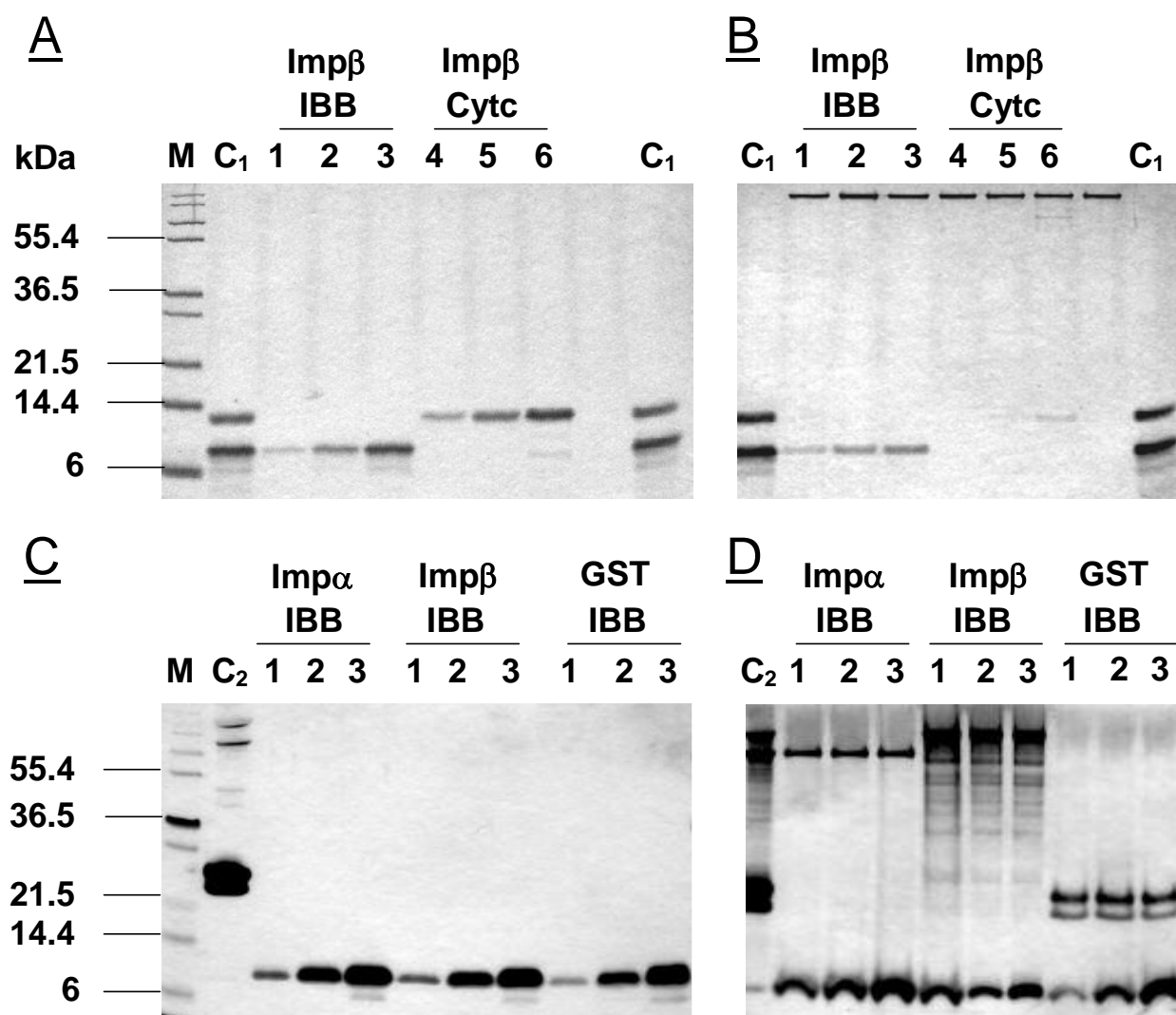
peptide to DNA did not give the effect we had expected, we examined the binding characteristics of IBB peptide.

Interaction of free and covalently coupled IBB peptide with importin β – Nuclear import assay

Interaction of the free IBB peptide with its intracellular target, namely importin β , was studied using recombinant importin β -GST fixed on glutathione-sepharose beads. In a first series of assays, we tested the interaction of either free IBB peptide or, as a control that of cytochrome c, with importin β (Figures 2A and 2B). The IBB peptide was detected in the bound fraction (Figure 2B), whereas cytochrome c was almost completely recovered in the unbound fraction (Figure 2A). This suggested that the synthetic IBB peptide was still able to interact with its nuclear

import receptor, namely importin β , as the hSRP1 α IBB domain described by Weis et al. [21].

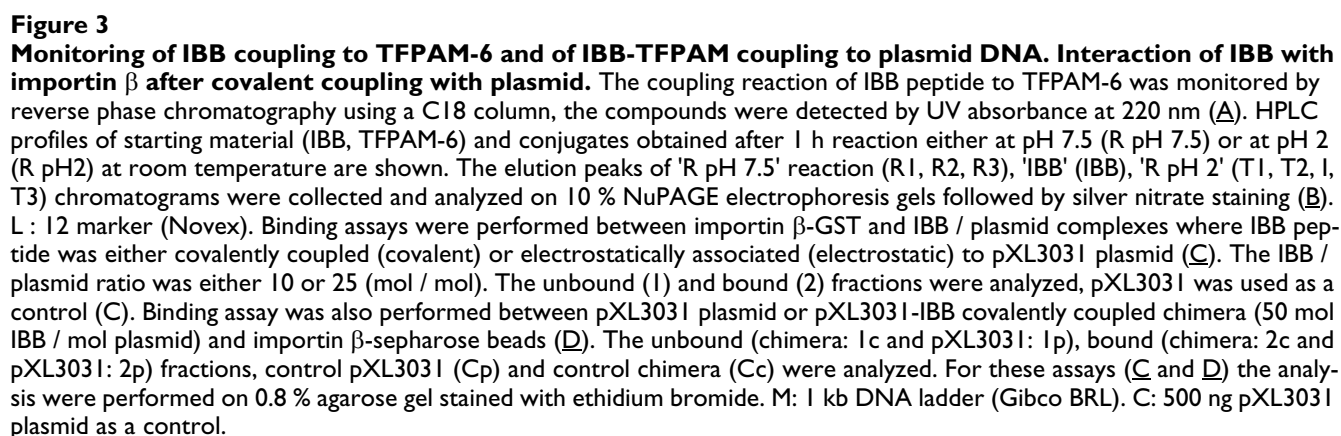
However, this interaction was shown to be non-specific in a series of control experiments as the IBB peptide also interacted with importin α and GST (Figures 2C and 2D). No interaction could be detected between the non-functionalized glutathione-sepharose beads and the IBB peptide (data not shown). Thus, the IBB peptide was shown to be able to interact with cytoplasmic receptors that mediate karyopheric proteins nuclear import, but not specifically.

**Figure 2**

Qualitative analysis of IBB peptide binding on importin α , β , and GST. Binding assays were performed between importin β -GST (Imp β) and IBB peptide (IBB) or cytochrome c (Cyt c) (**A** and **B**) and between importin α -GST (Imp α), importin β -GST (Imp β), or GST and IBB peptide (**C** and **D**). Importin-GST or GST were fixed on glutathione-sepharose beads and 1.25 μ g (1), 2.5 μ g (2) or 5 μ g (3) of IBB peptide, or 1.25 μ g (4), 2.5 μ g (5) or 5 μ g (6) of cytochrome c were applied to these beads. The unbound (**A** and **C**) and bound (**B** and **D**) fractions were analyzed on 10 % NuPAGE gels (Invitrogen) followed by Coomassie Blue staining (**A** and **B**) or silver nitrate staining (**C** and **D**). M: protein size ladder (Novex), C₁: 500 ng IBB peptide and 500 ng cytochrome c as a control, C₂: 500 ng GST, 500 ng importin α and 500 ng importin β as a control.

We then coupled IBB peptide to plasmid DNA. We first verified the formation of the IBB-TFPAM conjugate by HPLC (Figure 3A) followed by SDS-PAGE analysis of the eluted compounds (Figure 3B). At pH 2, the reaction cannot occur, peaks corresponding to non-coupled IBB (I) and TFPAM (T1, T2, T3) were observed. R1 and R2 peaks

that appeared only when the reaction was performed at pH 7.5 have migration properties on SDS-PAGE consistent with a 7.5 kDa-peptide. Considering their respective retention times, R1 was probably non-reacting IBB and R2 the TFPAM-IBB conjugate. We then added IBB-TFPAM to plasmid DNA and photoactivated the mixture. We were



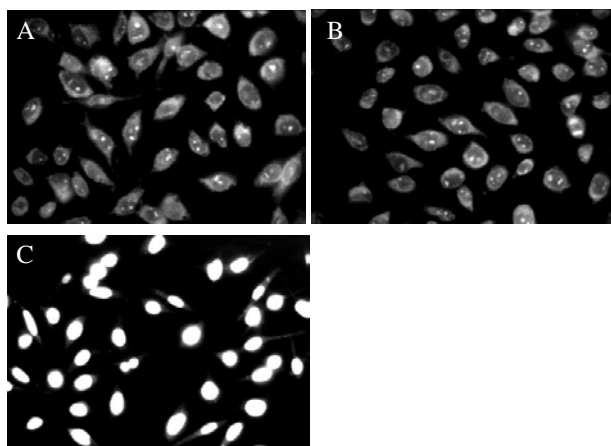


Figure 4
Fluorescence microscopy of nuclear import assay of IBB peptide-fluorescent plasmid in the digitonin-permeabilized HeLa cells system. Digitonin treated HeLa cells were incubated with 5 μ g fluorescent plasmid alone (A) or covalently coupled to IBB peptide at the ratio of 25 IBB peptide / plasmid (mol / mol) (B), or with 1 μ g fluorescent BSA-NLS (C) as a positive nuclear import control. The assay was performed for 30 min at 30°C in the presence of an energy regenerating system and 50 % (vol / vol) HeLa cytoplasmic extracts. Cells were observed with a 100 \times objective lens.

unable to purify the conjugate IBB-TFPAM-plasmid DNA from free IBB-TFPAM because IBB peptide bound to every support, dialysis membrane, plastic tube, etc. Every experiment was performed in low binding tubes to prevent non-specific interaction of the IBB peptide with the wall of the tubes. We have then no direct proof of the formation of the conjugate IBB-TFPAM-DNA except the fact that photoactivation of the complex IBB plasmid led to an inhibition of transfection. We have previously shown that the covalent coupling of ligands to plasmid DNA could cause inhibition of transfection, probably by transcriptional inactivation [26].

We next examined whether IBB peptide was still able to interact with its receptor when it was coupled to DNA. IBB peptide was coupled to DNA at two ratios and applied to importin β -containing beads. Bound and unbound fractions were analyzed by electrophoresis on agarose gel. As appeared in Figure 3C, at the ratio of 25 IBB peptides / plasmid (mol / mol), the covalent chimera were retained on importin β beads. However, this retention was also observed with non-covalently associated IBB / plasmid (Figure 3C). We therefore verified that plasmid DNA

alone (without IBB added) was unable to bind to these beads (Figure 3D). Taken together, these results proved that IBB was able to lead to the retention of the plasmid on importin β -conjugated beads even when non-covalent interactions occurred between the peptide and DNA. We hypothesized that covalent interactions occurred when photoactivation was applied, but we could not prove their existence due to the formation of strong non-covalent interactions between IBB and plasmid DNA.

Since IBB peptide associated with plasmid DNA was still able to bind importin β , we next evaluated whether IBB peptide was able to improve the nuclear import of plasmid DNA. We used the digitonin-permeabilized cells model with fluorescent plasmid. The IBB peptide was coupled to fluorescent plasmid at the molar ratio of 25 and incubated with digitonin-permeabilized HeLa cells. As shown in Figure 4, fluorescence was concentrated on the remaining cytoplasm and the nuclear envelope (Figure 4B), the staining pattern was the same with fluorescent plasmid alone, without IBB peptide covalent coupling (Figure 4A). No fluorescence was observed within cell nuclei.

It seemed that IBB peptide was still able to interact with its specific receptor, but despite this interaction no significant improvement of nuclear import of plasmid could be obtained.

Since IBB peptide was also able to interact non-specifically with other proteins, IBB-plasmid complexes might be sequestered by unspecific interactions with cytoplasmic proteins. Nevertheless, in an effort to obtain an improvement of gene transfer, we studied other formulations of DNA and IBB peptide and observed that IBB peptide / plasmid complexes formed by self assembly, without photoactivation, were able to increase gene transfer activity in particular conditions of transfection.

Gene transfer efficiency of self assembling complexes composed of IBB peptide / plasmid DNA / RPR120535 cationic lipid

We evaluated the effect of IBB peptide adjunction on transfection efficiency of DNA / cationic lipid complexes. These lipoplexes were prepared by mixing the lipofectant RPR120535 with plasmid DNA and IBB peptide in 150 mM NaCl in water and then diluted in serum containing cell culture medium. Without IBB, this formulation is known to lead to small lipoplexes and inefficient transfection [22–24].

The presence of IBB peptide greatly increased reporter gene expression. At ratios of 250 and 500 IBB peptide / plasmid DNA (mol / mol), i. e. 0.8 to 1.6 IBB peptide / plasmid DNA (w / w), we observed a 20-fold increase of

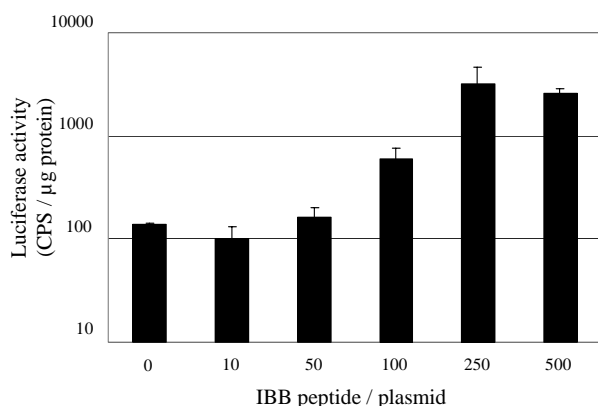


Figure 5
Transfection of HeLa cells using a ternary lipoplex. IBB peptide / plasmid / RPR120535 cationic lipid lipoplexes were prepared in 150 mM NaCl and diluted with serum-containing cell culture medium. The peptide was electrostatically associated to pXL3031 luciferase-encoding plasmid at a ratio varying from 0 to 500 IBB peptide / plasmid (mol / mol). Luciferase expression was evaluated 24 h after transfection.

luciferase gene expression (Figure 5). Enhancement of gene transfer efficiency depended on IBB peptide / plasmid DNA molar ratio: reporter gene expression increased with the amount of added IBB peptide. Transfection assays were also performed with lipoplexes prepared in water. In those assays, gene expression was increased 100-fold by adding 500 moles IBB peptide per mole plasmid DNA (data not shown).

We then wondered if this transfection enhancement could be attributed to a nuclear targeting of plasmid DNA by IBB peptide or to a change in the overall physico-chemical properties of plasmid DNA / RPR120535 lipoplexes.

Physico-chemical properties of the plasmid DNA / IBB complexes

The IBB peptide is globally basic, characterised by the presence of 17 R and K aminoacids, protonated at physiological pH, and of 10 acidic residues (D, E). Its charge at neutral pH is globally positive, for this reason it can potentially form complexes with negatively charged DNA molecules.

The formation of self-assembling IBB peptide / plasmid DNA complexes was examined by analysis of their electrophoretic mobility on agarose gel (Figure 6). At ratios of 100 and 250 IBB / plasmid (mol / mol), i. e. 0.3 and 0.8 IBB / plasmid (w / w), the migration of plasmid DNA was delayed. For ratios of 500 to 2000 IBB / plasmid (mol /

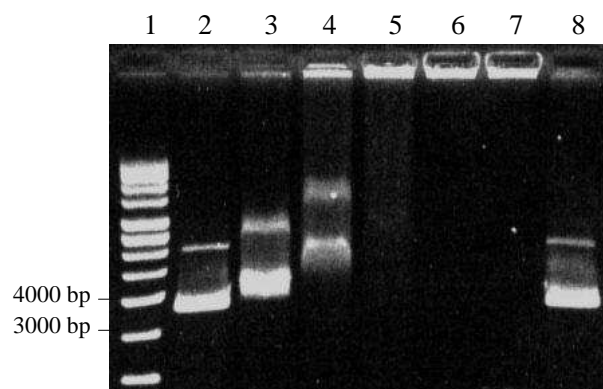


Figure 6
Complexation of plasmid DNA by IBB peptide. Complexation was allowed to proceed 15 min at room temperature in 50 mM Hepes buffer, pH 7.5. The migration of DNA was analyzed on a 0.8 % agarose gel stained with ethidium bromide. Lane 1: supercoiled DNA ladder (Gibco BRL), lane 2 and 8: pXL3031 plasmid (3700 bp), lane 3 to 7: IBB peptide / pXL3031 complexes at molar ratio of 100 (3), 250 (4), 500 (5), 1000 (6) and 2000 (7).

mol), i.e. 1.6 to 6 IBB / plasmid (w / w), no DNA migration occurred. This lack of migration indicates neutralization of nucleic acids by cationic IBB peptide and / or formation of large complexes that cannot migrate through the gel.

Lipoplexes size determination

The size of the ternary complexes was monitored using dynamic light scattering and the size distribution of the complex's populations was determined by size distribution processor analysis. When compared to binary DNA / cationic lipid complexes prepared in water, the addition of 250 mol of IBB peptide per mol plasmid DNA induced a marked size increase, leading to 3000 nm sized complexes (Figure 7A). In the case of 500 IBB / plasmid DNA (mol / mol), the lipoplexes were totally aggregated, as eye-observable aggregates could be seen (data not shown). In this case, lipoplex size cannot be precisely defined due to the detection limits of the nanosizer.

Lipoplexes prepared in water or in 150 mM NaCl and then diluted in serum-containing cell culture medium are known to be small and to lead to inefficient transfection [22–24]. On the contrary, preparing lipoplexes in bicarbonate buffer (20 mM NaHCO₃, 150 mM NaCl, pH 10), and diluting them in serum-containing cell culture medium leads to bigger lipoplexes and highly efficient transfection. The size of ternary IBB / DNA / RPR120535 complexes

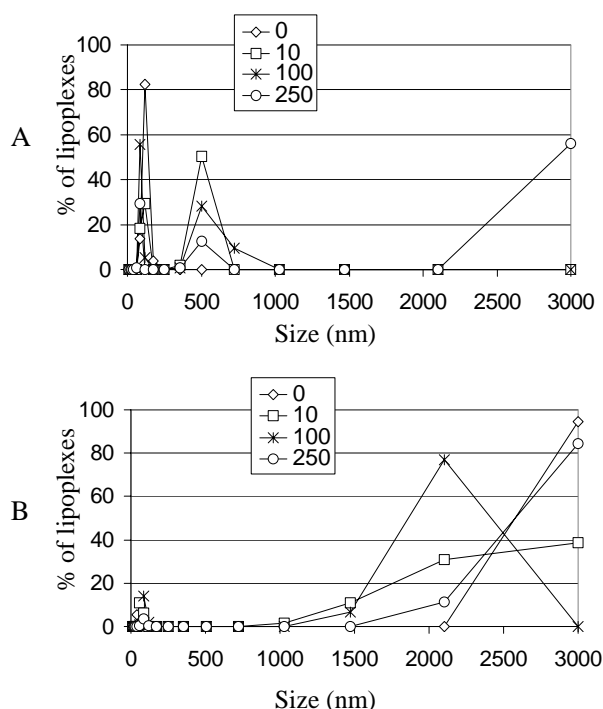


Figure 7
Size distribution of IBB peptide / plasmid / RPR120535 ternary lipoplexes. Lipoplexes were prepared at 0, 10, 100 and 250 IBB peptide / plasmid (mol / mol) by mixing plasmid with IBB peptide and then adding RPR120535 cationic lipid and diluting the preparation to the tenth either in water (A) or in NaCl 150 mM, 20 mM NaHCO₃, pH 10 (B). After 5 min equilibration at room temperature, lipoplex size was evaluated by the measure of dynamic light scattering at the angle of 90° for 10 min.

prepared in bicarbonate buffer (20 mM NaHCO₃, 150 mM NaCl, pH 10) were around 2000–3000 nm (Figure 7B), which was comparable to the size of IBB / plasmid DNA / RPR120535 lipoplexes prepared in water with 250 and 500 IBB / plasmid (mol / mol) (Figure 7A). Transfection with these ternary lipoplexes prepared in bicarbonate buffer was as efficient as transfection with binary plasmid DNA / RPR120535 complexes prepared in bicarbonate buffer (data not shown).

IBB peptide / plasmid / lipofectant complexes that exhibited an improvement of transfection clearly had different physico-chemical characteristics.

Discussion

In an aim to increase the nuclear targeting of plasmid DNA, we have covalently coupled importin α nuclear localization sequence, the IBB domain, to plasmids.

Covalent coupling of 50 to 500 IBB peptides per plasmid (mol / mol) led to a decrease in reporter gene expression. This inhibition of transfection was a consequence of the photoactivation since non-photoactivated IBB peptide / plasmid complexes of the same high ratio exhibited the same transfection activity as the control plasmid. This provides an indirect proof of the formation of covalent interactions between IBB-TFPAM and plasmid DNA. For lower peptide / plasmid molar ratio, we could not detect any increase in lipofectant-mediated transfection.

We used *in vitro* tests to evaluate IBB peptide interactions with the nuclear import machinery. The IBB peptide interacted with importin β , although non-specifically, since it also interacted with importin α and GST. In the cytoplasm, the IBB domain of importin α is never accessible: it is either associated with the NLS binding site of the same importin α [7,8], or with importin β . These two conformations should avoid this basic IBB from interacting with non-relevant cytoplasmic proteins. This could explain the interaction observed between IBB peptide and importin α . However, IBB interacted with GST protein; this interaction was not physiologically relevant and likely resulted from non-specific electrostatic association. In agreement with these results, the nuclear import either in the digitonin-permeabilized cell system or in transfection assays with fluorescent plasmid DNA was not increased when plasmid DNA was coupled with IBB peptide (data not shown): IBB peptide should interact with non-relevant cytoplasmic proteins before gaining access to the nuclear import machinery. However, fluorescence microscopy is not a highly sensitive method and plasmid DNA could also have entered the nuclei without being detectable. Another hypothesis is that IBB peptide was not functional.

Our strategy of using IBB sequence to enhance nuclear import of plasmid DNA was not demonstrated probably due to the propensity of IBB to interact non-specifically with irrelevant proteins. However, we hypothesized that this highly cationic peptide could associate with polyanionic plasmid DNA. We demonstrated that this association exists, as illustrated by the decrease or complete loss of plasmid agarose gel electrophoresis migration, depending on the IBB / plasmid ratio. Another indication of this self-association is the increase in lipoplex size when IBB peptide is used as an additive in the formulation.

We observed a 20-fold increase in gene expression when plasmid DNA was self-associated to IBB peptide prior to compaction with a cationic lipid such as RPR120535, as compared to transfection by binary plasmid / cationic lipid lipoplexes. This result was obtained in conditions known to lead to inefficient transfection, i.e. lipoplexes prepared in 150 mM NaCl and diluted in serum-containing cell culture medium. Serum is known to strongly inhibit gene transfer *in vitro*, through the formation of small sized DNA / cationic lipid complexes [22,23]. The IBB peptide seems to be responsible for the formation of large aggregates, which promote an efficient transfection. The same transfection enhancement has already been described when plasmid DNA is pre-compacted by cationic peptides derived from histone and protamine before complexing with a lipofectant [24]. As for the IBB peptide, the histone or protamine derived peptide promote the intracellular accumulation of fluorescent plasmid DNA in large cytoplasmic vesicles (data not shown) [24]. This previous data, in conjunction with the present results with IBB peptide, point to a general mechanism of action of cationic peptides-containing lipoplexes. Such ternary formulation displays high serum-resistant transfecting activity through the intracellular build-up of large aggregated complexes, which might have favored endosome-disrupting capacity.

Methods

Plasmid, proteins and peptide

Plasmid pXL3031 contains the enhancer-promoter from the immediate-early gene of cytomegalovirus (CMV), the engineered cytoplasmic luciferase gene (luc+) and the SV40 polyadenylation site. Luc+ is a high activity cytosolic form of luciferase obtained from pGL3 vectors (Promega). This pCOR plasmid (bearing a conditional origin of replication) was grown and purified as described [25]. Fluorescent plasmids were prepared as described previously [26]: a p-azido-tetrafluoro-benzyl-lissamine conjugate was synthesized and used to covalently associate fluorescent molecules to plasmid DNA by photoactivation (100 to 200 fluorophores per plasmid DNA).

The IBB peptide is composed of hSRP1 α 11–54 amino acids (human importin α 2, [20]), fused to a histidine tag (HHHHHH) and a myc-tag (EQKLISEEDL). Peptide [H]HHHHHHGEQKLISEEDLAARLHRFKNGKGD-STEMRRRRRIEVNVELRKAKDDQMLKRRNVSC [OH] was chemically synthesized and purified by reverse-phase chromatography (Dictagene S.A., Suisse). Lyophilized aliquots were maintained at -20°C, or dissolved in 50 mM HEPES buffer pH 7.5 (1 μ g/ μ l) containing 1 μ g/ml aprotinin, leupeptin and pepstatin and maintained at -80°C. Recombinant importin α -GST and importin β -GST were prepared as described previously [15].

Covalent coupling of IBB peptide to plasmid DNA

IBB peptide was covalently coupled to plasmid DNA using a previously described protocol [15]. Briefly, IBB peptide was incubated for 1 h at room temperature in 50 mM HEPES buffer pH 7.5 with the linker N-(4-azido-2,3,5,6 tetrafluorobenzyl)-6-maleimidyl hexanamide (TFPAM-6). The maleimide moiety of TFPAM-6 reacted with the thiol of the terminal cysteine of IBB peptide to form IBB-TFPAM. This reaction occurs only in a narrow range of pH, around pH 7, but at pH 2 the reaction does not occur. Reactions at pH 7.5 and at pH 2 were monitored by reverse-phase chromatography using a C18 Vydac – 238 TP 54 column (250 \times 4.6 mm), and an elution gradient of acetonitrile / water 0.1 % TFA (from 10 to 60 % acetonitrile in 30 min) with a flow rate of 1 ml / min. Eluted compounds were detected by UV absorbance at 220 nm. IBB-TFPAM was dialyzed 2 h at room temperature against 50 mM HEPES pH 7.5, using a Spectra / Por asymmetric cellulose ester dialysis membrane (MWCO 2,000 Da). Plasmid DNA and IBB-TFPAM were mixed, placed in a 96-well plate on an ice bath and subjected to photoactivation 15 min at 365 nm, under a sun lamp (Rad free UV lamp-365 nm, Schleicher and Schuell, Ecqueville, France). Plasmid DNA concentration was evaluated using the intercalating agent Picogreen (Molecular Probes) and peptide concentration was determined using Pierce BCA assay (Interchim).

Importin binding assay

Recombinant importin α -GST and importin β -GST were coupled to glutathione-sepharose 4B beads (Amersham Pharmacia Biotech) by incubation 30 min at room temperature of 1 μ g protein per 10 μ l of beads (10 μ g/ml final protein concentration in binding buffer: 20 mM HEPES pH 7, 150 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT and 0.1 % casaminoacids). The beads were collected by centrifugation and washed five times with binding buffer. Such a resin is called a functionalized resin.

For IBB peptide and cytochrome c binding assays: IBB peptide or cytochrome c were applied to importin α – and importin β -functionalized beads. For IBB / plasmid complexes binding assay: plasmid either covalently coupled or electrostatically associated to IBB peptide was applied to importin β -functionalized beads. For plasmid binding assay: plasmid was applied to importin β -functionalized beads.

After 1 h incubation at room temperature with soft agitation, the beads were collected by centrifugation and the supernatant removed (this supernatant is designated the "unbound fraction"). The beads were then washed five times with binding buffer. Proteins or complexes coupled to one volume of beads were eluted by heating 15 min at

80°C in one volume of elution buffer (0.1 % SDS, 0.1 M EDTA pH 8). The beads were collected by centrifugation and the supernatant was removed (this supernatant is called the "bound fraction"). The unbound and bound fractions were analyzed by gel electrophoresis. For IBB peptide and cytochrome c binding assay analysis, 10 % NuPAGE pre-cast gels (Invitrogen) were used, and then stained either by Coomassie Blue or silver nitrate staining [27]. For plasmid and IBB / plasmid binding assay analysis 0.8 % agarose gel in TBE buffer was used, and then stained with ethidium bromide.

Transfection studies

HeLa cells (ATCC CCL-2) were grown in complete cell culture medium, composed of Minimal Essential Medium (or MEM, GIBCO BRL) supplemented with 2 mM glutamine, 100 units / ml penicillin, 100 µg/ml streptomycin and 10 % foetal bovine serum. Cells were grown at 37°C in a 5 % CO₂ / air incubator.

For plasmid DNA expression studies, 24-well culture plates were seeded with 60,000 cells per well one day before transfection. Lipopolyamine RPR120535 [28] was used as a gene vector. Plasmids were diluted in 150 mM NaCl / water pH 6 or 150 mM NaCl / 20 mM NaHCO₃ / water pH 10 and mixed (vol / vol) with RPR120535 at the final concentration of 2.5 µg/ml plasmid DNA and 6 nmol RPR120535 / µg DNA. The complexes were allowed to form 15 min at room temperature and then diluted to the tenth in complete cell culture medium containing 10 % serum before addition to the cells (1 ml per well).

After 24 h at 37°C in a 5 % CO₂ / air incubator, the cells were washed twice with PBS and harvested with 200 µl of cell culture lysis reagent (Promega). Luciferase expression was quantified on 10 µl of centrifuged lysate supernatant using a luciferase assay kit (Promega). Light emission was measured in white 96-microwell plates (OptiPlate™ 96, Packard) using a Wallac Victor² microplate reader. Light emission, expressed in CPS (count per second), was normalized to the protein concentration of each sample, determined using Pierce BCA assay (Interchim).

Nuclear transport assay on digitonin permeabilized cells

Digitonin-permeabilized HeLa cells were prepared according to Adam et al. [29,30]. Cells grown to 90 % confluency on glass coverslips were immersed for 5 min on ice in 40 µg/ml ice-cold digitonin (Sigma) diluted in nuclear import buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM DTT, 1 µg/ml aprotinin, leupeptin and pepstatin) and then washed twice in cold nuclear import buffer. We then deposited a 50 µl drop of nuclear import buffer containing an ATP regenerating system (1 mM ATP, 0.5 mM GTP, 10 mM creatine

phosphate and 0.4 U / ml creatine phosphokinase), HeLa cytoplasmic extracts (~7 mg/ml final protein concentration), and 5 µg plasmid DNA labelled with lissamine (26), alone or complexed with IBB peptide (1 mol plasmid per 25 or 50 mol IBB). Nuclear transport was allowed to proceed for 30 min at 30°C. Coverslips were then rinsed twice with cold transport buffer, fixed in 2 % paraformaldehyde containing 0.1 % glutaraldehyde, and mounted on slides in PBS containing 50 % glycerol. Slides were fixed with nail polish and examined with a Zeiss Axiophot fluorescence microscope as described previously.

Particle size measurements

Plasmid DNA / IBB peptide complexes were prepared at molar ratios of 10, 25, 50, 100, 250, 500 (IBB peptide / plasmid DNA) in a final volume of 40 µl water. These complexes were then mixed (vol / vol) with RPR120535 at the molar ratio of 6 nmol RPR120535 / µg plasmid DNA. Complexes were allowed to form 15 min at room temperature and then diluted to the tenth in complete cell culture medium (final volume: 800 µl). Size analyses were performed on a Coulter N4-Plus nanosizer. After 3 min equilibration, sizes were evaluated by the measure of dynamic light scattering at an angle of 90° for 10 min.

Authors' contributions

MC carried out the entire study and drafted the manuscript. AS participated in the transfection studies. VE and DS conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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